PATENT
Attorney Docket No.: 025663-001310US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

ABREU, et al.

Application No.: 10/526,256

Filed: May 5, 2006

For: MUTATIONS IN NOD2 ARE ASSOCIATED WITH FIBROSTENOSING DISEASE IN PATIENTS WITH CROHN'S DISEASE

Customer No.: 20350

Confirmation No. 7450

Examiner: Goldberg, Jeanine Anne

Technology Center/Art Unit: 1634

DECLARATION UNDER 37 CFR § 1.131

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

1. We, Maria T. Abreu, Kent D. Taylor, Jerome I. Rotter, Stephan R. Targan and HuiyingYang declare as follows:

2. We are co-inventors of the above-referenced patent application.

3. We conceived of and reduced to practice the claimed invention in the United States *prior to* August 30, 2002, the filing date of 60/407, 391, from which the subject application claims priority.

4. Attached Exhibit A is a journal article that embodies the currently claimed invention. The journal article is entitled, "Mutations in NOD2 are associated with fibrostenosing disease in patients with Crohn's disease, by Maria T. Abreu, *et al.*, pages 679-688, Volume 123, Issue 3, (September 2002).

5. The Table of Contents of this September 2002 journal issue is attached as Exhibit B.

6. These Exhibits provide evidence of the conception of the invention and its reduction to practice. A manuscript, from which this journal article arose, was prepared prior to February 8, 2002. This is clear from the footnote on page 688 of the journal article, which states that the manuscript was received by the publisher on February 8, 2002.

7. In view of the foregoing, we respectfully submit that Exhibit A establishes that the claimed invention was conceived and reduced to practice prior to February 8, 2002.

8. We further declare that all statements made herein of our knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statement and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that any such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Signed: _____ Dated: 9/28/09
Maria T. Abreu

Signed: _____ Dated: 9/1/2009
Kent D. Taylor

Signed: _____ Dated: 9/1/09
Jerome I. Rotter

Signed: _____ Dated: 9-08-09
Stephan R. Targan

Signed: _____ Dated: _____
Huiying Yang

62030402 v1

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Signed: _____ Dated: _____

Maria T. Abreu

Signed: _____ Dated: _____

Kent D. Taylor

Signed: _____ Dated: _____

Jerome I. Rotter

Signed: _____ Dated: _____

Stephan R. Targan

Signed: Huiying Yang Dated: 6/2/09

Huiying Yang

62030402 v1

EXHIBIT A

GASTROENTEROLOGY 2002;123:679–688

Mutations in *NOD2* Are Associated With Fibrostenosing Disease in Patients With Crohn's Disease

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Background & Aims: The clinical manifestations of Crohn's disease (CD) are diverse, ranging from fibrostenosing small-bowel disease to colon-predominant inflammation. These distinctions may represent genetic, immunologic, and microbial heterogeneity. *NOD2* gene mutations in CD have been described recently and may alter innate immune responses. We hypothesized that *NOD2* mutations may be associated with distinct phenotypic expressions of CD. *Methods:* Two cohorts of consecutively identified patients referred to an inflammatory bowel disease center (n = 142 collected between 1993 and 1996; n = 59 collected between 1999 and 2001) were genotyped for 3 single nucleotide variants of *NOD2*—R675W, G881R, and 3020insC—and phenotyped for disease behavior, disease location, and serum immune markers. *Results:* Univariate analysis showed that CD-associated *NOD2* variants were significantly associated with fibrostenosing disease in each cohort ($P = 0.049$ and $P = 0.002$, respectively). When both cohorts were analyzed together, the association between *NOD2* variants and fibrostenosing disease was more significant ($P = 0.001$). These relationships were observed in both Jews and non-Jews. Forty-six percent of patients with fibrostenosing disease carried at least 1 of these alleles, compared with only 23.5% of patients without fibrostenosing disease (odds ratio, 2.8; 95% confidence interval, 1.6–5.2). Multivariate and conditioning analyses showed a primary association between *NOD2* allelic variants and fibrostenosing disease, but not with small-bowel disease. *Conclusions:* In this description of a genotype/phenotype correlation in CD patients and *NOD2* variants, data suggest that variation in this gene contributes to the occurrence of fibrostenotic CD of the small bowel.

Crohn's disease (CD) is a phenotypically heterogeneous disorder with diverse clinical manifestations. It may be characterized at multiple levels. Clinicians have characterized CD on the basis of the location of the disease (i.e., small bowel, colon, or both) or on the basis of complications of the disease (i.e., stricturing or perforating).[1–3] CD may also be classified by the expression of serum immune markers such as anti–*Saccharomyces cerevisiae* antibody (ASCA) and perinuclear antineutrophil cytoplasmic antibody (pANCA)[2–4] or by response to therapy, e.g., steroid dependent or infliximab responsive.[5–7] At the root of these disparate clinical manifestations lies an interplay of genetic, immunologic, and possibly microbial factors that culminates in distinct phenotypic expressions of the disease.

Animal models have provided novel insights into the pathogenesis of human inflammatory bowel disease (IBD) and identified candidate immunologic pathways that result in intestinal inflammation. These studies have also highlighted the need for commensal bacteria to unleash the host susceptibility.[8–12] Among the abnormalities identified in patients with CD is the finding of immunologic reactivity toward the individual's microbial flora, whereas healthy controls are immunologically tolerant to the indigenous flora.[13] Thus, IBD may result from an abnormal mucosal immune response to commensal bacteria or unidentified pathogens.

Sensing of bacterial products by the innate immune system is mediated by a family of receptors, toll-like receptors (TLRs), which activate the transcription factor nuclear factor κB (NF-κB) in response to pathogen-associated molecular patterns such as lipopolysaccharide (LPS). TLR4 is required for recognition of LPS.[14–17] We have recently described that intestinal epithelial cells

Abbreviations used in this paper: ASCA, anti–*Saccharomyces cerevisiae* antibody; CI, confidence interval; ELISA, enzyme-linked immunosorbent assay; EU, ELISA units; LPS, lipopolysaccharide; NF-κB; nuclear factor κB; OR, odds ratio; pANCA, perinuclear antineutrophil cytoplasmic antibody; SNP, single nucleotide polymorphism; TLR, toll-like receptor; UC, ulcerative colitis.

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express low levels of TLR4 and are LPS unresponsive,[18] suggesting that one mechanism by which the mucosal immune system is protected against chronic inflammation is down-regulation of TLR signaling molecules. Ogura et al.[19] have recently identified the *NOD2* gene. Its protein product is a member of the Apaf-1/Nod1 family of caspase-recruitment domain–containing proteins and is homologous to disease-resistance genes found in plants.[20] *NOD2* is expressed by monocytes and activates NF-κB in response to LPS. Several recent studies have identified a frameshift mutation in *NOD2* (3020insC) in patients with CD that results in a 33–amino acid truncation of the protein.[21–23] The frameshift mutation and an additional polymorphism of *NOD2* associated with CD are located in the leucine-rich region of the gene, suggesting that these rare alleles may affect the ability of NOD2 to interact with other intracellular proteins. Expression of this truncated mutant of *NOD2* in 293T cells dampens NF-κB activation in response to LPS.[22] Since the original identification of 3 allelic variants of *NOD2* associated with CD, mutational analysis in 453 patients with CD has identified an additional 27 rare mutations that account for 19% of all *NOD2* mutations.[24] More than 90% of all the Crohn's-associated *NOD2* mutations are located in the distal third of the gene, suggesting that these may also affect the function of *NOD2* with respect to bacterial recognition and signaling. Patients with a related syndrome, Blau's syndrome, characterized by granulomatous inflammation, have distinct mutations in *NOD2* within the nucleotide-binding domain of the gene.[25] These data support the notion that innate immunity is altered in patients with CD, resulting in abnormal immune responses to commensal or pathogenic bacteria.

We have previously described that the serum immune markers pANCA and ASCA are associated with distinct clinical phenotypes of CD.[2,3] Studies have shown that these antibodies recognize bacterial or yeast pathogen–associated molecular patterns.[26–29] Because *NOD2* regulates responses to bacterial products in monocytes, we hypothesized that carriage of *NOD2* mutants may be associated with specific clinical phenotypes of CD. The results of our studies reported herein show that mutations in *NOD2* occur more frequently in patients with stricturing, i.e., fibrostenotic, CD of the small bowel. Similar phenotypic findings have recently been described in 2 large European cohorts.[24,30] The data in our report strengthen this association and extend it to an American population. These results have important implications for the identification of host/microbial interactions that result in stricturing complications of CD.

Materials and Methods

Human Subjects

Two cohorts of patients were examined in this study. Both cohorts were consecutively identified as CD patients from an IBD referral center (Cedars–Sinai Medical Center Inflammatory Bowel Disease Center). The first cohort (n = 142) was ascertained between 1993 and 1996 and has been previously described.[2] The second cohort (n = 59) was collected between 1999 and 2001. Thus, the study population consisted of 201 consecutively ascertained patients evaluated by the Cedars–Sinai Medical Center Inflammatory Bowel Disease Center, with an established diagnosis of CD. A cohort of 175 patients with ulcerative colitis (UC) was used as an inflammatory disease control group. This study was reviewed and approved for human subject participation by the Cedars–Sinai Institutional Review Board and permitted the collection of clinical, serological, and genetic data from patients consenting to the study. Diagnosis of CD was defined by the presence of a combination of established features from at least 2 of the following categories: (1) clinical—perforating or fistulizing disease, obstructive symptoms secondary to small-bowel stenosis, or stricture; (2) endoscopic—deep linear or serpiginous ulcerations, discrete ulcers in normal-appearing mucosa, cobblestoning, or discontinuous or asymmetric inflammation; (3) radiographic—segmental disease (skip lesions), small-bowel or colon strictures, stenosis, or fistulae; or (4) histopathologic—submucosal or transmural inflammation, multiple granulomas, marked focal cryptitis, or focal chronic inflammatory infiltration within and between biopsies, or skip lesions, including rectal sparing in the absence of local therapy.

Phenotypic Analyses

Patients with CD were characterized as having fibrostenosing disease, internal-perforating disease, perianal fistulizing disease, or UC-like disease on the basis of previously described criteria.[1–3,31] Briefly, patients were considered to have fibrostenosing disease if they had documented persistent intestinal obstruction or required an intestinal resection for an intestinal obstruction. Internal perforating disease was recorded if patients had current or previous evidence of entero-enteric or entero-vesicular fistulae, intra-abdominal abscesses, or small-bowel perforation. Perianal perforating disease was recorded if patients had current or previous evidence of either perianal fistulae or abscesses or rectovaginal fistulae. Finally, UC-like disease was recorded if patients had current or previous evidence of left-sided colonic involvement, symptoms of bleeding or urgency, and crypt abscesses on colonic biopsies, as previously described.[3] Disease location was classified as small bowel, colon, or both on the basis of endoscopic, radiological, or pathologic studies. A panel of IBD physicians (M.T.A., E.A.V., L.Y.K., K.A.P., and S.R.T.) masked to the results of serological or genetic testing reached a consensus on phenotype on the basis of the clinical data.

Table 1. Primers and Probes

SNP	PCR		
	Forward primer	Reverse primer	TaqMan probes
5	5′-GGG TGG CTG GGC TCT TCT-3′	5′-CTC GCT TCC TCA GTA CCT ATG ATG-3′	5′FAM-CAT GGC TGG ACC C-MGBNFQ 5′TET-CAT GGC TGG ATC C-MGBNFQ
8	5′-GGC GGG ATG GAG TGG AA-3′	5′-CTG GCT GAG TGC CAG ACA TCT-3′	5′FAM-TGC TCC GGC GCC A-MGBNFQ 5′TET-CTG CTC TGG CGC CA-MGBNFQ
12	5′-CCA CCT CAA GCT CTG GTG ATC-3′	5′-GTT GAC TCT TTT GGC CTT TTC AG-3′	5′FAM-CTG TGT TGC CCC AGA A-MGBNFQ 5′TET-CTC TGT TGC GCC AGA-MGBNFQ
13	5′-CCT TAC CAG ACT TCC AGG ATG GT-3′	5′-TGT CCA ATA ACT GCA TCA CCT ACC T-3′	5′FAM-CCT TTC AAG GGG CCT-MGBNFQ 5-TET-CTT TCA AGG GCC TGC-MGBNFQ

Genotyping

By use of the software PrimerExpress 1.5 (PE Biosystems, Foster City, CA), the sequence information found in dbSNP (http://www.ncbi.nlm.nih.gov) for the *NOD2* R675W, G881R, and 3020insC mutations (also referred to as single nucleotide polymorphisms [SNP] 8, 12, and 13),[21] as well as SNP 5, the background allele for these mutations, was used to design genotyping assays with 5′-exonuclease technology,[32] also known as the TaqMan MGB assay (PE Biosystems). The MGB design adds a "minor groove binder" to the 3′ end of the TaqMan probes that increases the binding temperature of the probe and thus enables shorter probes to be used than in conventional TaqMan assays.[33] This has the effect of increasing the discrimination between the alleles in the assay.[34] Assays were performed by following the manufacturer's recommendations (PE Biosystems bulletin 4317594) in an ABI 7900 instrument. Genotyping was performed blinded to clinical status of the subjects (Table 1).

Serological Analyses

Serum ANCA expression and ANCA subtype characterization were performed by fixed neutrophil enzyme-linked immunosorbent assay (ELISA) as previously described.[35] Briefly, human peripheral blood neutrophils fixed with methanol were reacted with control and coded sera at a 1:100 dilution. Anti-human immunoglobulin G (IgG) (γ chain–specific) antibody (Jackson Immunoresearch Labs, Inc., West Grove, PA) conjugated to alkaline phosphatase was added to label neutrophil-bound antibody, and a colorimetric reaction was performed. Levels were determined relative to a standard consisting of pooled sera obtained from well-characterized pANCA$^+$ UC patients. Results were expressed as ELISA units (EU) per milliliter. ANCA$^+$ sera were further subtyped via indirect immunofluorescent staining to determine the ANCA neutrophil binding pattern, as previously described.[35] Sera showing the characteristic perinuclear highlighting and losing its characteristic staining pattern when treated with deoxyribonuclease were termed *pANCA*$^+$.[36] For the purposes of this study, patients were considered pANCA$^+$ if they were both positive for ANCA by-ELISA and lost perinuclear immunofluorescence staining with deoxyribonuclease treatment.

Sera were analyzed for ASCA expression in a blinded fashion by using a fixed ELISA assay.[2,37] Two patients in the second cohort did not undergo ASCA testing. High-binding polystyrene microtiter plates were coated with purified phosphopeptidomannans extracted from yeast *S. uvarum*, a subspecies of *S. cerevisiae*. Coded patient sera were diluted and added to the wells, followed by an enzyme-linked colorimetric reaction. Color development was proportional to concentrations of antibody present in the sera. Levels were determined and results expressed as EU per milliliter, relative to a standard, which was derived from a pool of patient sera with well-characterized CD found to have reactivity to this antigen. Sera showing ASCA IgG reactivity of >40 EU/mL or IgA reactivity of >20 EU/mL were termed ASCA$^+$. Serological assays were performed at Cedars–Sinai Medical Center and Prometheus Laboratories (San Diego, CA) by using identical methodology.

Statistical Analyses

To identify clinical features and immunologic traits that are associated with allelic variants of the *NOD2* gene, our study was designed to analyze 2 consecutively ascertained cohorts of patients with CD. The first cohort was used to explore the relationship of *NOD2* alleles to an array of clinical and serological variables and generate hypotheses. The second cohort was then used to confirm the specific hypotheses generated from analysis of the first cohort. To minimize the type I error and to maximize the statistical power, we permitted the significance of the associations in the first cohort to be less stringent ($P < 0.1$) and used the second, independent cohort to confirm the associations identified in the first cohort ($P < 0.05$). By avoiding a highly stringent correction for the number of variables examined in the first cohort, this strategy has the advantage of increasing the power to identify specific associations between *NOD2* and clinical variables, especially because some of these traits are known to be associated with each other (e.g., small-bowel involvement and ASCA expression).

Because the 3 rare alleles are independently associated with CD,[21] we analyzed each of them individually as well as combined. Statistical analysis was performed with SAS computer software (Version 6.10; SAS Institute, Inc., Cary, NC).[38] Quantitative variables are described as median (range) throughout. Nonparametric statistical tests were used to test differences of quantitative variables between 2 groups. The χ^2 or Fisher exact test (when the expected number was <5) was

used to evaluate associations between carriers and noncarriers of the rare alleles or between genotypes and categorical variables, such as type of IBD, disease location, disease behavior, and antibody positivity. Multivariate analysis was performed with the logistic regression model to test the association between genotypes and phenotypic variables that were significantly associated with *NOD2* variants from the univariate analyses. In addition, the Mantel–Haenszel stratified association test was performed for genotype and phenotype associations by controlling for potential confounding effects due to ethnic variation.[39] This stratified association test was also used to show whether the association between *NOD2* variants and a phenotype (e.g., fibrostenosing disease) was primary or secondary to other related phenotypes (e.g., small-bowel involvement).

Results

Patients With Crohn's Disease Have an Increased Frequency of Rare Allelic Variants of *NOD2*

An association between CD and allelic variants of *NOD2* has been previously described.[21–23] All 3 studies identified an association between CD and an insertion polymorphism in *NOD2*, 3020insC or 980fs (SNP 13), but only the Hugot et al. study[21] further identified 2 missense mutations, R675W (SNP 8) and G881R (SNP 12). We first wished to determine whether our North American CD referral patient population exhibited similar allelic variants of *NOD2* and could serve as a relevant population in which to study differences in phenotypic expressions associated with *NOD2* variant alleles. To address this question, cohort 1 (hypothesis generating) and cohort 2 (hypothesis confirming) (see Materials and Methods) were genotyped for all 3 variants—R675W (SNP 8), G881R (SNP 12), and 3020insC (SNP 13). The clinical characteristics of these 2 CD cohorts are shown in Table 2. In general, the first cohort had a higher percentage of patients with perforating and fibrostenotic complications of disease. These differences may be due to availability of improved therapy for CD in the second cohort or more severe CD in the first cohort. A cohort of UC patients was used as an inflammatory disease control group. Each of the 3 allelic variants of *NOD2* was significantly more frequent in patients with CD compared with UC (Table 3). As can be seen in Table 3, the frequency of each of the *NOD2* variants was extremely similar in each cohort of CD patients, supporting their combined use in the final analysis. The overall frequency of carriage of any *NOD2* allelic variant was 35% in CD patients, compared with 11% in UC patients ($P = 0.001$). Within the combined CD cohort, the frequency of homozygotes with the 3020insC mutation and of compound heterozygotes was 1% and 4%, respectively, whereas none of the UC patients had such a genotype. We conclude from these data that allelic variants of *NOD2* are associated with CD across diverse geographic and ethnically defined patient populations.

Table 2. Clinical Characteristics of CD Cohorts

Clinical characteristic	Cohort 1 (n = 142)	Cohort 2 (n = 59)	*P*
Sex (M/F)	79/63	33/26	0.969
Age of onset (*yr*)	22 (4–67)	22 (2–58)	0.6621
Ethnicity (Jew/non-Jew)	60/82	23/36	0.668
Disease location (%)			
Small bowel only	19.0	26.4	0.496
Colon only	20.4	20.8	
Small bowel and colon	60.6	52.8	
Perianal perforating (%)	35.9	28.8	0.332
Internal perforating (%)	47.2	23.7	0.002
Fibrostenosing disease (%)	59.9	30.5	0.001
UC-like (%)	39.4	22.0	0.018
pANCA positive (%)	19.7	12.5	0.295
ASCA positive (%)[a]	57.0	38.6	0.019

[a] Two patients in the second cohort did not undergo ASCA testing.

Mutations of *NOD2* Are Associated With Fibrostenosing Crohn's Disease

Patients with CD express diverse clinical phenotypes that may be due to differences in underlying genetic factors. We hypothesized that mutations in *NOD2* may be associated with specific CD-related clinical phenotypes. We further hypothesized that mutations in *NOD2* may be associated with specific CD-related serum immune markers. To test these hypotheses, we performed univariate analyses evaluating the association between *NOD2* allelic variants and predefined clinical characteristics, including age of onset, disease location, and disease phenotype (i.e., fibrostenosing disease, internal perforating disease, perianal fistulizing disease, or UC-like disease). Additionally, we tested the association between *NOD2* allelic variants and expression of the serum immune markers ASCA and pANCA. Univariate analysis showed that the CD-related *NOD2* variants were significantly associated with fibrostenosing disease in cohort 1 ($P = 0.049$) for the 3 allelic variants combined (Table 4). A positive association at a less stringent significance level ($P < 0.1$) was also observed with small-bowel involvement and younger age of onset, and a negative association was observed with UC-like disease in this cohort. With respect to serological markers, patients with the insertion mutation 3020insC were more likely to express ASCA ($P = 0.053$).

Based on our data in the first cohort, we hypothesized that NOD2 variants were positively associated with fibrostenosing Crohn's disease, small bowel involvement,

Table 3. Frequency of *NOD2* Allelic Variants in 2 Cohorts of Patients With CD (CD1 and CD2) and Ulcerative Colitis

Allelic variant	UC (n = 175)	CD1 (n = 142)	CD2 (n = 59)	Combined CD (n = 201)	*P* (UC vs. combined CD)
R675W (SNP 8)	5.7%	16.9%	15.3%	16.4%	0.001
G881R (SNP 12)	1.7%	12.0%	10.2%	11.4%	0.0001
3020insC (SNP 13)	3.4%	11.3%	11.9%	11.4%	0.004
Carriage of any allelic variant	10.9%	36.6%	32.2%	35.3%	0.001

ASCA positivity, and younger age of onset and negatively associated with UC-like disease. In cohort 2, we tested these specific hypotheses generated from cohort 1. As with cohort 1, cohort 2 demonstrated a significant association between NOD2 allelic variants and fibrostenosing disease ($P = 0.002$, with Bonferroni correction $P = 0.01$) (Table 4). When the 2 cohorts were analyzed together, the association between NOD2 variants and fibrostenosing disease was even more significant ($P = 0.001$) (Figure 1). These relationships were observed in both Jews and non-Jews. Approximately 46% of CD patients with fibrostenosing disease (Jews 52% vs. non-Jews 42%) have at least one of these rate alleles compared with only 23% (Jews 21.6% vs. non-Jews 25%) of CD patients without fibrostenosing disease (OR, 2.8; 95% CI, 1.56–5.18) (Figure 1). Of the 3 rare alleles, the frameshift mutation, 3020insC, demonstrated the greatest association with fibrostenosing disease (47% vs. 17%, *P* for cohorts combined = 0.006).

Table 4. Relationship of *NOD2* Allelic Variants and Clinical Phenotypes of CD in Cohort 1

		Qualitative trait %*NOD2* variant carriers			
Clinical phenotype	n	R675W (SNP 8)	G881R (SNP 12)	3020insC (SNP 13)	Carriage of any allelic variant
Small-bowel involvement					
Yes	113	19.5%	11.5%	14.2%	40.7%
No	29	6.9%	13.8%	0.00%	20.7%
P		0.081	0.494	0.04	0.063
Perianal perforating					
Yes	51	11.8%	11.8%	13.7%	35.3%
No	91	19.8%	12.1%	9.9%	37.4%
P		0.248	0.839	0.547	0.747
Internal perforating					
Yes	67	13.4%	16.4%	11.9%	37.3%
No	75	20.0%	8.0%	10.7%	36.0%
P		0.346	0.178	0.91	0.96
Fibrostenosing					
Yes	85	18.8%	14.1%	15.3%	43.5%
No	57	14.0%	8.8%	5.3%	26.3%
P		0.389	0.458	0.084	0.049
UC-like					
Yes	56	17.9%	10.7%	5.4%	30.4%
No	86	16.3%	12.8%	15.1%	40.7%
P		0.822	0.736	0.076	0.22
pANCA positive					
Yes	28	17.9%	14.3%	7.1%	32.1%
No	114	16.7%	11.4%	12.3%	37.7%
P		0.82	0.793	0.394	0.529
ASCA positive					
Yes	81	18.5%	9.9%	16.1%	38.3%
No	61	14.8%	14.8%	4.9%	34.4%
P		0.467	0.234	0.053	0.744
		Quantitative trait, median (*range*)			
Age of onset (*yr*)					
Carrier of *NOD2* variant					
Yes		22 (6–67)	22 (4–62)	19 (10–50)	20 (4–67)
No		22 (4–63)	22 (4–67)	22 (4–67)	22 (4–63)
P		0.715	0.937	0.074	0.238

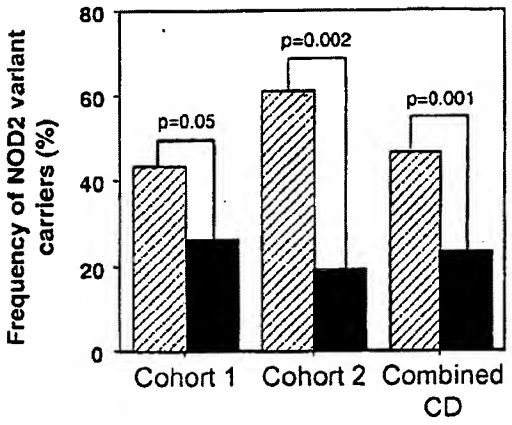

Figure 1. Rare allelic variants of *NOD2* are associated with fibrostenosing Crohn's disease. Two cohorts of Crohn's disease patients were genotyped for 3 allelic variants of *NOD2*. Carriage of these allelic variants was more frequent in Crohn's disease patients with fibrostenosing disease (▨) compared with those who did not have fibrostenosing disease (■).

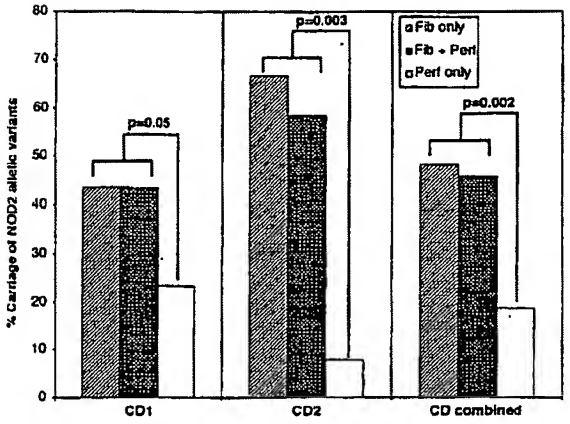

Figure 3. Comparison of *NOD2* allelic frequencies in patients with fibrostenosing disease compared with perforating disease. Patients were separated by the presence of fibrostenosing disease with (Fib + perf) or without (Fib only) perforating complications and compared with patients with perforating complications and without evidence of fibrostenosis (Perf only). Patients with evidence of fibrostenosis were significantly more likely than those with purely perforating disease to carry *NOD2* allelic variants in each of the cohorts and in the combined cohorts.

We next analyzed the risk of fibrostenosing disease in CD patients carrying homozygous mutations or compound heterozygous mutations in *NOD2*. Compared with patients who were not carriers of *NOD2* mutations, patients who were carriers of 2 mutations in *NOD2* were significantly more likely to have fibrostenosing disease (85% vs. 43%; odds ratio [OR], 7.4; 95% confidence interval [CI], 1.9–28.9; $P = 0.004$) (Figure 2). Patients who were carriers of a single *NOD2* mutation were also significantly more likely to have fibrostenosing disease

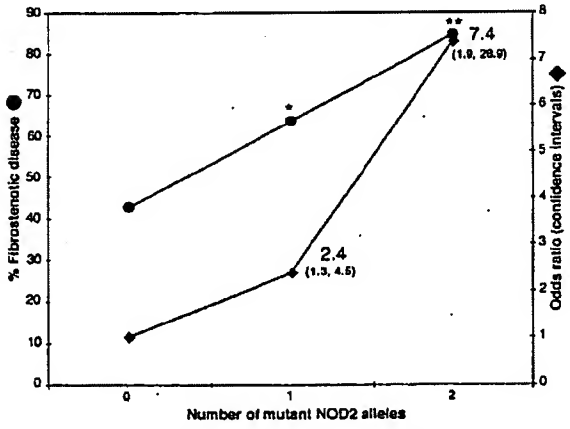

Figure 2. Frequency of fibrostenosing complications in patients with *NOD2* allelic variants. On the basis of genotyping for the 3 rare alleles of *NOD2*, patients could be described as carrying 0, 1, or 2 rare alleles (*x* axis). The left *y* axis shows the frequency of fibrostenosing complications (●); $*P = 0.008$, $**P = 0.004$ compared with 0 alleles. The right *y* axis shows the odds ratio (◆), with 95% confidence intervals in parentheses.

when compared with patients who were not carriers of *NOD2* mutations (64% vs. 43%; OR, 2.37; 95% CI, 1.26–4.47; $P = 0.008$). The patients with fibrostenosing disease in these 2 cohorts could be characterized as having only fibrostenosing disease or both fibrostenosing and perforating disease, because these 2 phenotypes often occur in the same patient. The percent of *NOD2* variants in patients with fibrostenosing disease only was 48.3%, which was similar to that seen in patients with both fibrostenosing and perforating complications (46.0%; $P = 0.8$). When we compared patients with fibrostenosing disease with those patients described as having perforating disease only (perianal or internal), the percentage of carriage of *NOD2* allelic variants in patients with fibrostenosing disease (with or without perforating complications) (46.6%) was much greater than that seen in patients with only perforating complications (18.6%; $P = 0.002$) (Figure 3).

Studies performed on large European cohorts of CD patients have shown an association between carriage of *NOD2* allelic variants and small-bowel involvement[30,40] and a negative association with colonic involvement[24] and younger age of onset.[24,30] In our cohorts, a trend toward small-bowel involvement was seen in the first cohort (40.7% vs. 20.7%) (Table 4) and again in the second cohort (35.7% vs. 23.5%) (Table 5). When the 2 cohorts were analyzed together, small-bowel involvement was found to be significantly associated with carriage of *NOD2* variants (39.4% vs. 21.7%; $P = 0.036$).

Table 5. Relationship of *NOD2* Allelic Variants and Clinical Phenotypes of CD in Cohort 2

Clinical phenotype	n	Qualitative trait %*NOD2* variant carriers			
		R675W (SNP 8)	G881R (SNP 12)	3020insC (SNP 13)	Carriage of any allelic variant
Fibrostenosing					
Yes	18	22.2%	22.2%	27.8%	61.1%
No	41	12.2%	4.9%	4.9%	19.5%
P		0.315	0.048	0.018	0.002
Small-bowel involvement					
Yes	42	19.1%	9.5%	14.3%	35.7%
No	17	5.9%	11.8%	5.9%	23.5%
P		0.22	0.828	0.288	0.354
UC-like					
Yes	13	7.7%	15.4%	7.7%	23.1%
No	46	17.4%	8.7%	13.0%	34.8%
P		0.399	0.489	0.593	0.432
ASCA positive					
Yes	22	9.1%	13.6%	13.6%	31.8%
No	35	17.1%	8.6%	11.4%	31.4%
P		0.4	0.542	0.735	0.956
		Quantitative trait, median (*range*)			
Age of onset (*yr*)					
Carrier of *NOD2* variant					
Yes		27 (10–58)	26 (7–33)	17 (13–35)	22 (7–58)
No		19 (2–55)	20 (2–58)	24 (2–58)	22 (2–55)
P		0.332	0.9	0.566	0.981

As expected, a negative trend was seen between carriage of *NOD2* variants and UC-like CD for the cohorts combined (OR, 0.37; 95% CI, 0.12–1.09; $P = 0.071$). The second cohort did not, however, show an association between *NOD2* variants and ASCA positivity. In addition, although the younger age of onset seemed to be associated with the 3020insC allele of *NOD2* in the first cohort ($P = 0.074$), this association was not significant in the second cohort. In the combined CD cohorts, this association showed a borderline significance ($P = 0.062$). In multivariate analysis, all variables with at least borderline significance ($P < 0.1$) in either cohort were tested simultaneously for their association with *NOD2* allelic variants by using logistic regression. As shown in Table 6, the only phenotype that was significantly associated with *NOD2* ($P < 0.05$) was fibrostenosing disease (OR, 2.8; 95% CI, 1.3–6.0). In summary, these data show that fibrostenosing disease is independently associated with *NOD2* allelic variants regardless of ethnic background and other clinical phenotypes.

Table 6. Multivariate Analysis in the Combined Cohort for 5 Phenotypic Variables

Clinical phenotypes	OR	95% CI	*P*
Fibrostenosing disease	2.8	1.3–6.0	0.011
Small bowel involvement	1.3	0.5–3.4	0.561
UC-like	0.9	0.4–1.7	0.658
ASCA positive	0.7	0.3–1.3	0.250
Age of onset	1.0	0.9–1.0	0.874

NOTE. Multivariate analysis was performed for the 5 phenotypes that demonstrated an association by univariate analyses. Of the variables tested, only fibrostenosing disease demonstrated an independent association with NOD2 allelic variants.

Because fibrostenosing disease is more likely to occur in patients with small-bowel involvement, we stratified patients on the basis of small-bowel involvement to further address the primary association between fibrostenosing disease and *NOD2* variants. Among patients with small-bowel involvement, 26.4% of patients who did not have fibrostenosing disease (n = 53) had a *NOD2* variant, whereas a much greater percentage (46.1%) of patients who had fibrostenosing disease (n = 102) had a *NOD2* variant ($P = 0.017$). A similar trend was observed among patients without small-bowel involvement ($P = 0.05$), and the combined analysis conditioning on small-bowel involvement yielded a significance level of 0.009. However, after controlling for fibrostenosing disease, small-bowel involvement was not associated with *NOD2* variants ($P = 0.63$). This result agrees with the results from the logistic regression analysis and suggests that the association between fibrostenosing disease and *NOD2* variants is independent of small-bowel involvement, but the observed small-bowel association with *NOD2* is secondary to the presence of fibrostenosing disease.

Discussion

CD is a multigenic disorder with diverse clinical manifestations. Several population-based studies have described an association of *NOD2* gene mutations in CD.[21–23] This study describes a genotype/phenotype association for *NOD2* allelic variants in CD. Specifically, we describe an association between the presence of *NOD2* mutations and small-bowel stricturing CD. Both the genetic association and its phenotypic association with fibrostenosis was observed in Jews and non-Jews with similar frequency. Our findings are consistent with those of Lesage et al.[24] and Ahmad et al.,[30] who described a genotype/phenotype association between *NOD2* variants and fibrostenosing disease in 2 large series of European patients. The Ahmad et al. study did not, however, find an association between *NOD2* variants and fibrostenosing disease that was independent of an association with small-bowel disease.[30] The finding of a CD subtype in patients carrying *NOD2* mutations provides biological evidence for the *NOD2* gene in the pathogenesis of CD and lends further support for *NOD2* as a CD susceptibility gene. Alternatively, these findings may be due to the effect of a neighboring gene in linkage disequilibrium with *NOD2*. The *NOD2* gene is also the first that has been described to be associated with fibrostenosing CD. Basic and translational studies will need to explore whether CD-associated *NOD2* mutations directly alter the path to fibrogenesis in response to small-bowel inflammation.

In designing this study, we explored a variety of previously described clinical phenotypes within CD[2,3] and their association with *NOD2* allelic variants. A major strength of our study lies in the ability to analyze 2 independent cohorts of CD patients with similar carriage of *NOD2* allelic variants. By setting a less stringent significance level in the first cohort, we were able to increase our power to detect clinical associations with *NOD2* mutations. The second cohort was then used to confirm the associations found in the first cohort and reduce type I error. The consistency of the *NOD2* genetic and phenotypic association in our cohorts, as well as in the European cohorts,[24,30] speaks strongly to the specific role of *NOD2* mutations in the development of fibrostenosing complications. Recently, other groups have described that *NOD2* mutations are associated primarily with ileal disease.[24,30,40] In our conditioning analysis, carriage of *NOD2* mutations was no more likely in patients with small-bowel involvement without stricturing complications (26.4%) than in CD overall. By contrast, patients with small-bowel involvement and stricturing complications were significantly more likely to have carriage of *NOD2* allelic variants (46.1%; $P = 0.017$). We, therefore, believe that mutations in *NOD2* are not associated just with small-bowel involvement, but rather with the subset of patients whose small-bowel disease becomes fibrostenotic. Other groups have also identified a younger age of onset in patients with *NOD2* mutations.[24,30] Although we did not find a similar association, it is likely that our study did not have the power to detect this smaller association.

In addition to showing an association between *NOD2* mutations and fibrostenosis, our data show that *NOD2* mutations are not associated with perforating complications of CD. Indeed, even in patients who can be described as having both fibrostenotic complications (e.g., small-bowel obstruction) and perforating complications (e.g., perianal fistula), these patients are genetically similar to those with fibrostenosis only (Figure 3). These data suggest that *NOD2* contributes to the pathogenesis of fibrostenosis regardless of other superimposed complications, such as perforations. Little is known about the genetic or immunologic factors resulting in fibrostenosis in CD. Compared with Crohn's patients requiring surgery for perforating complications of the disease, patients with fibrostenotic disease have a longer interval between surgeries.[31,41,42] Studies have shown that fibroblasts isolated from strictures of patients with CD produce significantly more type III collagen than fibroblasts isolated from nonstrictured CD lamina propria.[43,44] Collagen production by intestinal fibroblasts is regulated by transforming growth factor β1, transforming growth factor β2, platelet-derived growth factor, and interleukin 1.[45,46] We have previously described that high levels of ASCA and expression of both IgG and IgA subtypes are highly associated with fibrostenosing disease and multiple surgeries.[2] ASCA is itself a heritable trait and is expressed by unaffected family members of ASCA$^+$ CD patients.[4,47] Linkage studies have shown that ASCA levels are linked with the major histocompatibility complex on chromosome 6, but not with the IBD1 locus (*NOD2* gene region) on chromosome 16.[48] Because fibrostenosing disease occurs more commonly in the small bowel and is associated with the serum immune marker ASCA,[2] we examined the association of *NOD2* mutations with ASCA positivity. We did not identify a significant association between *NOD2* variants and expression of ASCA. These data are consistent with our previous negative linkage results with the IBD1 locus[48] and suggest that the factors downstream of *NOD2* that result in fibrostenosis are distinct from those resulting in ASCA positivity.

In vitro studies have shown that expression of the truncated mutant of *NOD2* (3020insC or 980fs) results in diminished LPS responsiveness.[22] The functional phenotype of the 2 missense mutations in *NOD2* (R675W and G881R) and the additional 27 rare mutations with respect to LPS responsiveness is not clear but may result in other defects in innate immunity.[24] Although all 3 allelic variants were independently associated with CD (Table 3), carriage of the truncation mutation of *NOD2* was most strongly correlated with fibrostenosing disease. One model to reconcile diminished LPS responsiveness with *NOD2* mutations and the pathogenesis of CD is a defective response to pathogenic or commensal organisms resulting in a chronic infection or an aberrant immune response. Because *NOD2* is an intracellular protein primarily expressed by monocytes, mutations in *NOD2* may predispose the susceptible host to a chronic infection with an intracellular pathogen.[49,50] Given the diversity of microbes in the gut, it is also possible that *NOD2* variants may increase proinflammatory responses to specific bacteria or bacterial products other than LPS. Because we have described an association between fibrostenosing disease and *NOD2* variants, we hypothesize that the immune response distal to a mutation in *NOD2* shifts T cells toward transforming growth factor β cytokine production and increases collagen deposition by smooth muscle cells and fibroblasts in the intestine. At present, little other than surgical strategies can be offered to patients with fibrostenosing complications of CD. The results of our studies permit basic and translational researchers to identify links between *NOD2* and stricturing CD that may improve therapy for this group of patients.

References

1. Gasche C, Scholmerich J, Brynskov J, D'Haens G, Hanauer SB, Irvine EJ, Jewell DP, Rachmilewitz D, Sachar DB, Sandborn WJ, Sutherland LR. A simple classification of Crohn's disease: report of the Working Party for the World Congresses of Gastroenterology, Vienna 1998. Inflamm Bowel Dis 2000;6:8–15.
2. Vasiliauskas EA, Kam LY, Karp LC, Gaiennie J, Yang H, Targan SR. Marker antibody expression stratifies Crohn's disease into immunologically homogeneous subgroups with distinct clinical characteristics. Gut 2000;47:487–496.
3. Vasiliauskas EA, Plevy SE, Landers CJ, Binder SW, Ferguson DM, Yang H, Rotter JI, Vidrich A, Targan SR. Perinuclear antineutrophil cytoplasmic antibodies in patients with Crohn's disease define a clinical subgroup. Gastroenterology 1996;110:1810–1819.
4. Vermeire S, Peeters M, Vlietinck R, Joossens S, Den Hond E, Bulteel V, Bossuyt X, Geypens B, Rutgeerts P. Anti-*Saccharomyces cerevisiae* antibodies (ASCA), phenotypes of IBD, and intestinal permeability: a study in IBD families. Inflamm Bowel Dis 2001;7:8–15.
5. Munkholm P, Langholz E, Davidsen M, Binder V. Frequency of glucocorticoid resistance and dependency in Crohn's disease. Gut 1994;35:360–362.
6. Faubion WA Jr, Loftus EV Jr, Harmsen WS, Zinsmeister AR, Sandborn WJ. The natural history of corticosteroid therapy for inflammatory bowel disease: a population-based study. Gastroenterology 2001;121:255–260.
7. Taylor KD, Plevy SE, Yang H, Landers CJ, Barry MJ, Rotter JI, Targan SR. ANCA pattern and LTA haplotype relationship to clinical responses to anti-TNF antibody treatment in Crohn's disease. Gastroenterology 2001;120:1347–1355.
8. Schultz M, Sartor RB. Probiotics and inflammatory bowel diseases. Am J Gastroenterol 2000;95:S19–S21.
9. Rath HC, Ikeda JS, Linde HJ, Scholmerich J, Wilson KH, Sartor RB. Varying cecal bacterial loads influence colitis and gastritis in HLA-B27 transgenic rats. Gastroenterology 1999;116:310–319.
10. MacDonald TT, Pettersson S. Bacterial regulation of intestinal immune responses. Inflamm Bowel Dis 2000;6:116–122.
11. French N, Pettersson S. Microbe-host interactions in the alimentary tract: the gateway to understanding inflammatory bowel disease. Gut 2000;47:162–163.
12. Veltkamp C, Tonkonogy SL, De Jong YP, Albright C, Grenther WB, Balish E, Terhorst C, Sartor RB. Continuous stimulation by normal luminal bacteria is essential for the development and perpetuation of colitis in Tg(epsilon26) mice. Gastroenterology 2001;120:900–913.
13. Duchmann R, Kaiser I, Hermann E, Mayet W, Ewe K, Meyer zum Buschenfelde KH. Tolerance exists towards resident intestinal flora but is broken in active inflammatory bowel disease (IBD). Clin Exp Immunol 1995;102:448–455.
14. da Silva Correia J, Soldau K, Christen U, Tobias PS, Ulevitch RJ. Lipopolysaccharide is in close proximity to each of the proteins in its membrane receptor complex: transfer from cd14 to tlr4 and md-2. J Biol Chem 2001;276:21129–21135.
15. Bowie A, O'Neill LA. The interleukin-1 receptor/Toll-like receptor superfamily: signal generators for pro-inflammatory interleukins and microbial products. J Leukoc Biol 2000;67:508–514.
16. Zhang FX, Kirschning CJ, Mancinelli R, Xu XP, Jin Y, Faure E, Mantovani A, Rothe M, Muzio M, Arditi M. Bacterial lipopolysaccharide activates nuclear factor-kappaB through interleukin-1 signaling mediators in cultured human dermal endothelial cells and mononuclear phagocytes. J Biol Chem 1999;274:7611–7614.
17. Jiang Q, Akashi S, Miyake K, Petty HR. Lipopolysaccharide induces physical proximity between CD14 and toll-like receptor 4 (TLR4) prior to nuclear translocation of NF-kappa B. J Immunol 2000;165:3541–3544.
18. Abreu MT, Vora P, Faure E, Thomas LS, Arnold ET, Arditi M. Decreased expression of Toll-like receptor-4 and MD-2 correlates with intestinal epithelial cell protection against dysregulated proinflammatory gene expression in response to bacterial lipopolysaccharide. J Immunol 2001;167:1609–1616.
19. Ogura Y, Inohara N, Benito A, Chen FF, Yamaoka S, Nunez G. Nod2, a Nod1/Apaf-1 family member that is restricted to monocytes and activates NF-kappaB. J Biol Chem 2001;276:4812–4818.
20. Inohara N, Ogura Y, Chen FF, Muto A, Nunez G. Human Nod1 confers responsiveness to bacterial lipopolysaccharides. J Biol Chem 2001;276:2551–2554.
21. Hugot JP, Chamaillard M, Zouali H, Lesage S, Cezard JP, Belaiche J, Almer S, Tysk C, O'Morain CA, Gassull M, Binder V, Finkel Y, Cortot A, Modigliani R, Laurent-Puig P, Gower-Rousseau C, Macry J, Colombel JF, Sahbatou M, Thomas G. Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. Nature 2001;411:599–603.
22. Ogura Y, Bonen DK, Inohara N, Nicolae DL, Chen FF, Ramos R, Britton H, Moran T, Karaliuskas R, Duerr RH, Achkar JP, Brant SR, Bayless TM, Kirschner BS, Hanauer SB, Nunez G, Cho JH. A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. Nature 2001;411:603–606.
23. Hampe J, Cuthbert A, Croucher PJ, Mirza MM, Mascheretti S, Fisher S, Frenzel H, King K, Hasselmeyer A, MacPherson AJ, Bridger S, van Deventer S, Forbes A, Nikolaus S, Lennard-Jones

JE, Foelsch UR, Krawczak M, Lewis C, Schreiber S, Mathew CG. Association between insertion mutation in NOD2 gene and Crohn's disease in German and British populations. Lancet 2001;357:1925–1928.
24. Lesage S, Zouali H, Cezard JP, Colombel JF, Belaiche J, Almer S, Tysk C, O'Morain C, Gassull M, Binder V, Finkel Y, Modigliani R, Gower-Rousseau C, Macry J, Merlin F, Chamaillard M, Jannot AS, Thomas G, Hugot JP. CARD15/NOD2 mutational analysis and genotype-phenotype correlation in 612 patients with inflammatory bowel disease. Am J Hum Genet 2002;70:845–857.
25. Miceli-Richard C, Lesage S, Rybojad M, Prieur AM, Manouvrier-Hanu S, Hafner R, Chamaillard M, Zouali H, Thomas G, Hugot JP. CARD15 mutations in Blau syndrome. Nat Genet 2001;29:19–20.
26. Cohavy O, Bruckner D, Gordon LK, Misra R, Wei B, Eggena ME, Targan SR, Braun J. Colonic bacteria express an ulcerative colitis pANCA-related protein epitope. Infect Immun 2000;68:1542–1548.
27. Cohavy O, Harth G, Horwitz M, Eggena M, Landers C, Sutton C, Targan SR, Braun J. Identification of a novel mycobacterial histone H1 homologue (HupB) as an antigenic target of pANCA monoclonal antibody and serum immunoglobulin A from patients with Crohn's disease. Infect Immun 1999;67:6510–6517.
28. Eggena M, Cohavy O, Parseghian MH, Hamkalo BA, Clemens D, Targan SR, Gordon LK, Braun J. Identification of histone H1 as a cognate antigen of the ulcerative colitis-associated marker antibody pANCA. J Autoimmun 2000;14:83–97.
29. Sendid B, Colombel JF, Jacquinot PM, Faille C, Fruit J, Cortot A, Lucidarme D, Camus D, Poulain D. Specific antibody response to oligomannosidic epitopes in Crohn's disease. Clin Diagn Lab Immunol 1996;3:219–226.
30. Ahmad T, Armuzzi A, Bunce M, Mulcahy-Hawes K, Marshall SE, Orchard TR, Crawshaw J, Large O, de Silva A, Cook JT, Barnardo M, Cullen S, Welsh KI, Jewell DP. The molecular classification of the clinical manifestations of Crohn's disease. Gastroenterology 2002;122:854–866.
31. Greenstein AJ, Lachman P, Sachar DB, Springhorn J, Heimann T, Janowitz HD, Aufses AH Jr. Perforating and non-perforating indications for repeated operations in Crohn's disease: evidence for two clinical forms. Gut 1988;29:588–592.
32. Livak K. Allelic discrimination using fluorogenic probes and the 5'-nuclease assay. Genet Anal 1999;14:143–149.
33. Kutyavin I, Lukhtanov E, Gamper H, Meyer R. Oligonucleotides with conjugated dihydropyrroloindole tripeptides: base composition and backbone effects on hybridization. Nucleic Acids Res 1997;25:3718–3723.
34. Kutyavin I, Afonina I, Mills A, Gorn V, Lukhtanov E, Belousov E, Singer M, Walburger D, Lokhov S, Gall A, Dempcy R, Reed M, Meyer R, Hedgpeth J. 3'-Minor groove binder-DNA probes increase specificity at PCR extension temperatures. Nucleic Acids Res 2000;28:655–661.
35. Saxon A, Shanahan F, Landers C, Ganz T, Targan S. A distinct subset of antineutrophil cytoplasmic antibodies is associated with inflammatory bowel disease. J Allergy Clin Immunol 1990; 86:202–210.
36. Vidrich A, Lee J, James E, Cobb L, Targan S. Segregation of pANCA antigenic recognition by DNase treatment of neutrophils: ulcerative colitis, type 1 autoimmune hepatitis, and primary sclerosing cholangitis. J Clin Immunol 1995;15:293–299.
37. Vermeire S, Joossens S, Peeters M, Monsuur F, Marien G, Bossuyt X, Groenen P, Vlietinck R, Rutgeerts P. Comparative study of ASCA (anti-*Saccharomyces cerevisiae* antibody) assays in inflammatory bowel disease. Gastroenterology 2001;120: 827–833.
38. SAS/STAT user's guide. Version 6.12. SAS Institute Inc., Cary, NC, 1990.
39. Schlesselman J. Case-control studies: design, conduct, analysis. New York, NY, Oxford University Press, 1982.
40. Cuthbert AP, Fisher SA, Mirza MM, King K, Hampe J, Croucher PJ, Mascheretti S, Sanderson J, Forbes A, Mansfield J, Schreiber S, Lewis CM, Mathew CG. The contribution of NOD2 gene mutations to the risk and site of disease in inflammatory bowel disease. Gastroenterology 2002;122:867–874.
41. Lautenbach E, Berlin JA, Lichtenstein GR. Risk factors for early postoperative recurrence of Crohn's disease. Gastroenterology 1998;115:259–267.
42. Aeberhard P, Berchtold W, Riedtmann HJ, Stadelmann G. Surgical recurrence of perforating and nonperforating Crohn's disease. A study of 101 surgically treated patients. Dis Colon Rectum 1996;39:80–87.
43. Stallmach A, Schuppan D, Riese HH, Matthes H, Riecken EO. Increased collagen type III synthesis by fibroblasts isolated from strictures of patients with Crohn's disease. Gastroenterology 1992;102:1920–1929.
44. Pucilowska JB, Williams KL, Lund PK. Fibrogenesis. IV. Fibrosis and inflammatory bowel disease: cellular mediators and animal models. Am J Physiol Gastrointest Liver Physiol 2000;279: G653–G659.
45. Assche GV. Can we influence fibrosis in Crohn's disease? Acta Gastroenterol Belg 2001;64:193–196.
46. McKaig BC, Hughes K, Tighe PJ, Mahida YR. Differential expression of TGF-beta isoforms by normal and inflammatory bowel disease intestinal myofibroblasts. Am J Physiol Cell Physiol 2002;282:C172–C182.
47. Sutton CL, Yang H, Li Z, Rotter JI, Targan SR, Braun J. Familial expression of anti-*Saccharomyces cerevisiae* mannan antibodies in affected and unaffected relatives of patients with Crohn's disease. Gut 2000;46:58–63.
48. Yang H, Taylor K, Lin Y, Targan S, Rotter J. Magnitude of anti-*Saccharomyces cerevisiae* antibody (ASCA) expression is linked in Crohn's disease families to the major histocompatibility complex (MHC) region (abstr). Gastroenterology 2000;118(suppl): A339.
49. Zumla A, James DG. Granulomatous infections: etiology and classification. Clin Infect Dis 1996;23:146–158.
50. Chamberlin W, Graham DY, Hulten K, El-Zimaity HM, Schwartz MR, Naser S, Shafran I, El-Zaatari FA. Review article: *Mycobacterium avium* subsp. *paratuberculosis* as one cause of Crohn's disease. Aliment Pharmacol Ther 2001;15:337–346.

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